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Killing and Replacing Queen-Laid Eggs: Low Cost of Worker Policing in the Honeybee

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ABSTRACT: Worker honeybees, *Apis mellifera*, police each other's reproduction by killing worker-laid eggs. Previous experiments demonstrated that worker policing is effective, killing most (~98%) worker-laid eggs. However, many queen-laid eggs were also killed (~50%) suggesting that effective policing may have high costs. In these previous experiments, eggs were transferred using forceps into test cells, mostly into unrelated discriminator colonies. We measured both the survival of unmanipulated queen-laid eggs and the proportion of removal errors that were rectified by the queen laying a new egg. Across 2 days of the 3-day egg stage, only 9.6% of the queen-laid eggs in drone cells and 4.1% in worker cells were removed in error. When queen-laid eggs were removed from cells, 85% from drone cells and 61% from worker cells were replaced within 3 days. Worker policing in the honeybee has a high benefit to policing workers because workers are more related to the queen's sons (brothers, $r = 0.25$) than sister workers' sons (0.15). This study shows that worker policing also has a low cost in terms of the killing of queen-laid eggs, as only a small proportion of queen-laid eggs are killed, most of which are rapidly replaced.

Keywords: policing, cost benefit, recognition errors, egg, *Apis mellifera*, honeybee.

Introduction

Within insect societies there is extensive potential for conflict over reproduction (Ratnieks and Reeve 1992; Bourke and Franks 1995; Ratnieks et al. 2006). One important area of conflict is the production of males (Bourke and Franks 1995). Since males arise from unfertilized eggs, workers can normally contribute to male production, as in most species workers retain ovaries but cannot mate (Bourke 1988). In a bee, ant, or wasp colony, the proportion of the reared males that are workers' sons varies greatly among species (Wenseleers and Ratnieks 2006a). One factor of importance in causing this variation is variation in the extent of egg policing, the killing of worker-

laid eggs either by the queen (queen policing) or by workers (worker policing; Wenseleers and Ratnieks 2006a; 2006b). Several factors affect whether worker policing is favored by natural selection (Ratnieks and Wenseleers 2008). On relatedness grounds it is favored when the workers are more related to the sons of their mother queen, or queens, than to other workers' sons (Ratnieks 1988; Pamilo 1991; Crozier and Pamilo 1996; Wenseleers and Ratnieks 2006a). In species with single-queen colonies, this occurs when the effective paternity frequency of the mother queen is greater than 2 (Ratnieks 1988; Foster and Ratnieks 2001c). Worker policing can also be favored at effective paternity frequencies below 2 when it helps cause a female-biased sex allocation ratio (Foster and Ratnieks 2001b) or enhances colony productivity (Ratnieks 1988) or when policing workers themselves lay eggs (selfish worker policing; Stroeymeyt et al. 2007; Bonckaert et al. 2011; Zanette et al. 2012).

The honeybee, *Apis mellifera*, has a high paternity frequency (Tarpy and Page 2001; Tarpy and Nielsen 2002; Tarpy et al. 2004) and an effective system of worker policing (Wenseleers and Ratnieks 2006b). Most experimentally transferred worker-laid eggs, 98%–99%, are killed within 1 day in a colony with a queen (Ratnieks and Visscher 1989; Ratnieks 1990b; 1993), and only 0.12% of the adult males reared in colonies with a queen are workers' sons (Visscher 1989). Effective policing also acts as a deterrent against attempting to reproduce (Wenseleers and Ratnieks 2006b), and only ~0.01%–0.1% of the workers in a colony with a queen have active ovaries (Ratnieks 1993) versus ~36% in a queenless colony (Miller and Ratnieks 2001). However, it is estimated that these few egg-laying workers in a queenright colony could lay up to 25–50 eggs per day, 7% of the male eggs (Visscher 1996), which would result in a substantial proportion of the colony's male production if not policed (Ratnieks 1993; Visscher 1996).

Studies examining the policing of worker-laid eggs in honeybee colonies usually compare the survivorship of

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eggs that have been experimentally transferred from a queenless colony with egg-laying workers into test cells in an unrelated discriminator colony with similarly manipulated and unrelated queen-laid eggs taken from drone cells (i.e., male eggs). The results typically show significantly greater and more rapid killing of worker-laid eggs than queen-laid eggs. However, many of the queen-laid eggs are also killed. In European honeybees, *A. mellifera*, the proportion of queen-laid eggs still remaining in drone cells ranges from ~45% after 6 h (Beekman and Oldroyd 2005) to 55% after 24 h (Ratnieks 1990b) and 61% after 24 h (Ratnieks and Visscher 1989). Similar results have been found in other honeybee species, with 30% remaining after 24 h in *Apis florea* (Halling et al. 2001) and 25% after 20 h in *Apis cerana* (Oldroyd et al. 2001), and also in vespine wasps, which, like honeybees, have open hexagonal cells so that eggs can easily be checked. In *Vespula vulgaris* and *Vespa crabro*, 67% and 64% of the queen-laid eggs remained after 16 h, respectively (Foster and Ratnieks 2001a; Foster et al. 2002), and in *Vespula germanica* 53% survived for the first 24 h (Bonckaert et al. 2008).

These studies on the above six species with worker policing all found that a large proportion of queen-laid eggs were killed. Taken at face value, this implies that worker policing has a high cost due to the killing of queen-laid eggs. Although policed eggs are eaten in *A. mellifera*, which will reduce any waste of resources invested in the eggs themselves, the rearing of brood within cells in the brood area, which is thermoregulated at ~35°C (Seeley 1985), will be less efficient with more empty cells. This is because for the same number of offspring, a larger area of comb would have to be thermoregulated (Ratnieks 1990a). Alternatively, the high mortality of queen-laid eggs may be due to the experimental procedures used, which were designed to compare the survivorship of queen-laid versus worker-laid eggs under controlled conditions rather than to make absolute estimates of egg survival. In the honeybee studies, eggs were transferred using forceps, which damages eggs (Wegener et al. 2010). In addition, eggs may be less well placed in their new cells than if directly laid by a queen and are normally relocated into an empty comb, which may result in greater egg removal due to the reluctance of honeybees to rear small patches of brood (F. L. W. Ratnieks, personal observation). In the wasp and hornet studies, similar manipulations were made, except that eggs were removed from their original cell by cutting the paper comb so the egg remained attached to a small piece of paper that was then glued into the test cell.

The aim of this study was to investigate the cost of worker policing in the honeybee due to the removal of queen-laid eggs. To do this, we collected two types of data. First, we measured the mortality of unmanipulated queen-laid eggs in worker and drone cells. Second, we determined

the time taken by the queen to lay a new egg in a worker or drone cell from which an egg had been experimentally removed and how this was affected by the time after laying at which the original queen-laid egg was removed.

Material and Methods

Study Organisms, Sites, and Basic Methods

Experiments were conducted in June and July 2008 at an apiary of the Laboratory of Apiculture and Social Insects, University of Sussex, England, and in April and May 2009 at an apiary near the city of Graz, Austria. During these periods, colonies in these apiaries were naturally rearing large numbers of drones (males), with the queens readily laying eggs in the large-diameter hexagonal drone cells used to rear males.¹ The 2008 experiments were conducted near the end of the main drone-rearing season. To ensure that the experimental colonies would adequately care for any male eggs laid by the queens, they had been prevented from rearing drones before the experiment by giving them only combs of worker-sized cells and by removing the few drone pupae being reared in the few drone cells available. The 2009 experiments were conducted at the beginning of the main drone rearing season so that there was no need to prevent colonies from rearing drones before data collection. Thus, in both study years, the experimental colonies had a high incentive to rear drones.

Six colonies were used in experiments 1 and 2, the 2008 trial being carried out using three *Apis mellifera mellifera* colonies in England and the 2009 trial using three *Apis mellifera carnica* colonies in Austria. Experiment 3 used three *A. m. carnica* colonies in Austria. The use of two subspecies and locations was to increase the generality of the results rather than as a controlled comparison between subspecies. The study colonies were relatively pure representatives of their respective subspecies as shown by wing morphometry (Ruttner 1996; *A. m. mellifera*: cubital index = 1.81 ± 0.04 [mean \pm SE], $n = 100$ [colonies 1 and 2], $n = 99$ [colony 3]; *A. m. carnica*: cubital index = 2.85 ± 0.12 [mean \pm SE], $n = 100$ [colonies 4, 5, and 6]). The great majority of workers in the experimental colonies had the body color typical of their subspecies (gray, *A. m. carnica*; black, *A. m. mellifera*). All colonies were of similar size, housed in two hive boxes, a deep

¹ Mated *Apis mellifera* queens lay fertilized eggs in worker cells and unfertilized eggs in drone cells (Ratnieks and Keller 1998). Fertilized eggs, which are heterozygous at the sex determination locus, are female. Most are reared into workers and a few, in royal cells, into queens. Homozygotes are diploid males (Beye et al. 2003), which are killed as young larvae (Woyke 1963). Normal haploid males develop from unfertilized eggs (Beye et al. 2003), which are normally laid in drone cells by both queens and laying workers (Page and Erickson 1988; Ratnieks 1993).

Langstroth in England and a Steirisches Einheitsmaß in Austria, each box containing 10 frames of comb. A queen excluder was placed between the two boxes to confine the queen to the lower box. In experiment 1, brood frames were moved into the upper box during hive inspections so that the brood area also extended above the queen excluder.

Experiment 1: Survival of Queen-Laid Eggs

The aim of experiment 1 was to measure the survival of unmanipulated queen-laid eggs. We transferred one empty frame each of worker cells and drone cells from above to below the queen excluder to allow the queen to lay eggs. We checked these combs every 24 h, and when the queen had laid a patch of eggs, we removed the frame to the laboratory to inspect it under good illumination and to record and map the presence or absence of eggs in a subset of the cells. We then replaced the frame into the brood area above the queen excluder to prevent additional egg laying by the queen. This procedure was carried out twice per colony in each study location. Frames were moved only within a colony, never between colonies. Each subset (area) of cells we monitored (one subset per frame: mean = 838.2 cells, range = 592–1,333 cells, $n = 22$ subsets, data for worker cells of one colony excluded) was a rectangular patch of ~20 to 25 rows with ~40 cells on one side of the frame. In these patches, 221 to 708 cells contained an egg (mean = 501.7) and 87 to 906 cells contained no egg (mean = 336.5). To help relocate cells on subsequent inspections, the leftmost cell in every fifth row received a colored pin. The presence or absence of an egg was recorded on paper printed with a hexagonal pattern. Counting was performed as quickly as possible, within 15–30 min, to reduce the possibility of egg dehydration. Because egg laying by the queen may have taken place any time in the preceding 24 h, the postlaying ages of the eggs when checked were 0–24 (first inspection) and then 24–48 and 48–72 h on subsequent daily inspections. Honeybee eggs hatch after slightly over 3 days (Harbo and Bolten 1981), and only during the egg stage do workers discriminate between workers' sons and queen's sons (Ratnieks and Visscher 1989). In total, 59,400 cell inspections were made. In addition to the removal of queen-laid eggs, the inspections also allowed us to obtain data on the presence of eggs newly laid by workers in previously empty cells on the second and third inspections.

Because we made daily inspections of cells, we were unable to observe the fate of queen-laid eggs from the moment of laying. On the first inspection we were able to observe eggs laid in the previous 24 h. The three inspections, therefore, enabled us to quantify the removal of eggs over a 2-day period. For some eggs this would

have been mainly the first 2 days after laying, for others mainly the last 2 days, or something in between. We did not make more frequent egg counts as this would have required the study hives to be opened multiple times per day, which would have caused greater disturbance to the bee colony and required the test frames to be out of the hive for longer periods. Also, more frequent inspections would not have solved the principal problem of observation in time intervals. We made three daily inspections because the egg stage lasts ~3 days. Using the data on egg survival over 2 days, we estimated survival rate over the full 3 days using a simple model: having calculated the decrease of eggs after 24 and 96 h with a linear model, we divided the two values by 2 since only half of the eggs were present between 0 and 24 h (eggs were being laid by the queen) and between 72 and 96 h (larvae hatched from the eggs). However, between 0 and 24 h, the queen was not only laying eggs but could also replace removed eggs. According to our experiments, approximately 80% of eggs aged 24 h were replaced within 1 day. For simplicity, we took 80% for both, eggs in worker and drone cells. But since eggs were not removed at 0 h but between 0 and 24 h, on average only half of them, that is, approximately 40%, could have been replaced. Finally, we corrected for all percentages after every interval since, for example, a decrease of 6% of eggs in drone cells from inspection 1 to inspection 2 reduces to 5.85% when the percentage of eggs remaining at inspection 1 is not 100% but 97.48%.

Experiment 2: Replacement of Eggs in Worker and Drone Cells by the Queen

The aim of experiment 2 was to quantify the laying of replacement eggs in cells from which an egg had been experimentally removed. We placed an empty frame of worker cells with two 10 × 7-cm patches of drone cells, ~140 drone cells per side, below the queen excluder. When the queen had laid eggs, we then removed a small proportion of the eggs in the frame, 10 to 40 eggs each from worker and drone cells, and returned the frame below the queen excluder. To help relocate cells from which an egg had been experimentally removed on subsequent inspections, the leftmost cell in every row received a colored pin. We then checked these cells 24 and 48 h later to determine whether they contained a newly laid egg.

Experiment 3: Replacement of Eggs at Different Time Periods after Egg Removal

The aim of experiment 3 was to determine whether the timing of egg removal after laying affects the probability of egg replacement. Here we used the same method as in experiment 2, except that the test comb had only drone

cells. We removed 10 eggs per frame and returned the frame below the queen excluder. After a further 24 and 48 h, we removed an additional 10 eggs from the same patch. These eggs were now 24–48 and 48–72 h old, respectively. Each of these groups of 10 empty cells was checked every 24 h for another 2 days to quantify the laying of new eggs.

Statistical Analysis

Data were analyzed and plotted in R 2.10.1 (R Development Core Team 2009). To avoid pseudoreplication and to control for variation between colonies or cells, we used generalized linear mixed effects models for all statistical tests (Bolker et al. 2009; Zuur et al. 2009). For this purpose, the lmer function of the lme4 package was applied (Bates and Maechler 2010). Generally, data were binomially distributed apart from the time data in experiment 2, which were Poisson distributed. Where appropriate, we treated “colony” and/or “cell” as random effects and factors such as “subspecies,” “sex,” or “day” as fixed effects. We then compared random intercept models with random intercept and slope models using likelihood ratio tests and selected the model according to Zuur et al. (2009). Initially, interactions between fixed effects were included but non-significant interactions were removed in the final model. For data visualization we used the ggplot2 package (Wickham 2009). All grand means and grand percentages in this article are weighted by sample size and structure. Data sets of this article are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.g4052> (Kärcher and Ratnieks 2014).

Results

Experiment 1: Survival of Queen-Laid Eggs

Only a small proportion of queen-laid eggs were removed (fig. 1a, cumulative percentages). In total, 6.0% and 9.6% of the eggs in drone cells ($n = 18,447$) and 2.1% and 4.1% in worker cells ($n = 14,664$) were removed within 24 and 48 h, respectively, with the removal rate from drone cells being significantly higher ($P = 1.05 \times 10^{-7}$, $n = 33,111$; fig. 1). The *Apis mellifera carnica* colonies removed fewer eggs within 48 h (drone cells: $8.8\% \pm 5.8\%$, worker cells: $3.6\% \pm 1.5\%$, mean \pm SE) than the *Apis mellifera mellifera* colonies (drone cells: $10.4\% \pm 3.2\%$, worker cells: $4.7\% \pm 1.1\%$, $P = 2.98 \times 10^{-6}$, $n = 33,111$). The removal rate of queen-laid eggs from drone cells was significantly greater in the first 24-h monitoring period, when the eggs were aged 0–24 h at the start, than in the next 24-h period (6% vs. 3.6%, $P = 8.97 \times 10^{-14}$, $n = 12,298$; fig. 1). There was no such difference for queen-laid eggs

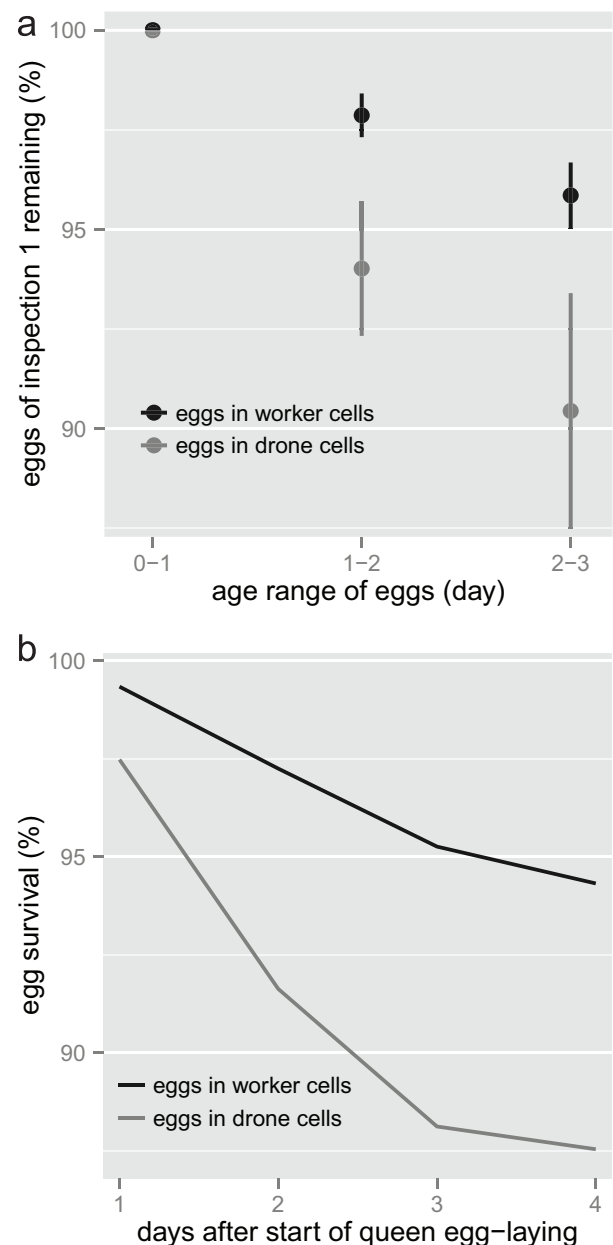


Figure 1: a, Experiment 1. Natural survival rate of queen-laid eggs in drone and worker cells over 2 days of the 3-day egg stage. Percentages are cumulative, dots represent means, and vertical lines represent standard errors. The black and gray dot at 100% represents the initial number of eggs at the age of 0–1 day, at which time a fraction of the queen-laid eggs would already have been removed by workers and largely replaced by the queen. Note the different decrease of the percentage of eggs in worker or drone cells until hatching. b, Experiment 1. Inferred, natural survival rate of queen-laid eggs in drone and worker cells during the entire 3 days of egg development according to our model. With “egg survival” we refer only to the survival of an individual during egg stage until a larva hatched from it, which happened 3–4 days after the start of queen egg laying. Lines represent estimated means of cumulative percentage decreases.

in worker cells (2.1% vs. 2%, $P = .0798$, $n = 9,776$; fig. 1).

Based on the data for removal over 2 days, our model indicates that 5.7% of the eggs in worker cells and 12.5% of the eggs in drone cells would have been removed during the full 3-day egg stage (fig. 1b). After the start of queen egg-laying 99.3%, 97.3%, 95.3%, and 94.3% of eggs in worker cells would have survived (or replaced again between 0 and 24 h when the comb was below the queen excluder) for 24, 48, 72, and 96 h. Of queen-laid eggs in drone cells 97.5%, 91.6%, 88.1%, and 87.5% would still be present after 24, 48, 72, and 96 h (fig. 1b).

Experiment 1: Additional Results: Laying and Survival of Worker-Laid Eggs

When checking cells, we occasionally found eggs in cells that had been empty on the previous day (one in a worker cell and 14 in drone cells), indicating that they were worker-laid eggs since the queen was confined below the queen excluder. The percentage of worker-laid eggs was higher in *A. m. mellifera* (0.7252% of previously empty drone cells, $n = 13$ eggs, and 1,503 drone cells) than in *A. m. carnica* (0.0572%, $n = 1$ egg, and 1,540 drone cells) ($P = .0284$, $n = 3,043$). There was no correlation between the proportion of worker-laid eggs appearing in drone cells and the proportion of queen-laid eggs that were policed in drone cells ($P = .5864$, $n = 12$ drone combs). Worker-laid eggs that had already survived 0–24 h in previously empty drone cells ($n = 9$) from inspection 1 to inspection 2 had a high survival rate over the next 24 h (46% weighted mean, 78% arithmetic mean, seven of nine eggs), that is, from inspection 2 to inspection 3. This was not significantly different to the survival of 0–24-h-old queen-laid eggs in drone cells during their subsequent 24 h (94%, $n = 6,149$), that is, from inspection 1 to inspection 2 ($P = .199$, $n = 6,158$). For these comparisons, only male eggs in drone cells were used because only one newly laid egg was found in a worker cell. In total, 14 worker-laid eggs were found in drone cells, 13 in cells that had not previously contained a queen-laid egg and one in a cell from which a queen-laid egg had been removed. Four of these 13 eggs were found when queen-laid eggs were already 48–72 h old. The other nine were found when queen-laid eggs were 24–48 h old so that we could track them for 1 day, to show that seven (46%) survived over the next 24 h. These nine worker-laid eggs were found in 3,029 drone cells containing no egg at first inspections (6,149 drone cells contained a queen-laid egg). Thus, after 24 h, there were approximately three worker-laid eggs per 1,000 empty drone cells. The actual egg-laying rate by workers would have been higher due to the egg removal before the daily inspection (Visscher 1996).

Experiment 1: Unexplained Result: Low Survival of Eggs in Worker Cells in One Colony

In one study colony in Austria, the proportion of queen-laid eggs removed from worker cells was much higher than in the other colonies. Although most of these eggs, 93.5%, survived to 24–48 h, only 71.3% survived to 48–72 h. The proportion of queen-laid eggs removed from drone cells, however, was very similar to the proportion removed in the other five colonies. At the end of the experiment, we noted that this colony had a much lower worker population than the other colonies and was not building up in population as expected. Therefore, we excluded the data for queen-laid eggs in worker cells from all analyses and figures of this article. However, this did not affect our results and, in particular, the conclusion that the proportion of queen-laid eggs that are killed is low. Including the data for eggs in worker cells of this colony would have raised the proportion of queen-laid eggs removed by workers to only 8.3% versus 4.1% mentioned above.

Experiment 2: Replacement of Eggs in Worker and Drone Cells by the Queen

Eggs were more likely to be replaced by the *A. m. carnica* ($86.5\% \pm 5.5\%$, mean \pm SE) than *A. m. mellifera* study colonies ($63.3\% \pm 17.0\%$; $P = .0252$, $n = 290$; fig. 2) within 3 days after egg removal. However, there was no

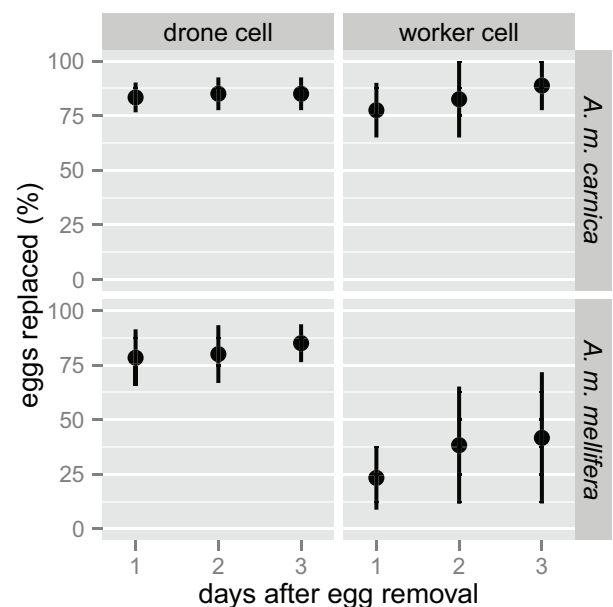


Figure 2: Experiment 2. Cumulative replacement, by the queen laying new eggs, of eggs removed at the age of 0–1 day from drone and worker cells in *Apis mellifera carnica* and *Apis mellifera mellifera*. Dots represent means. Vertical lines represent standard errors.

significant difference in the probability of an egg being replaced in a drone cell ($85.0\% \pm 5.1\%$) versus a worker cell ($60.5\% \pm 20.4\%$; $P = .6534$, $n = 290$, fig. 2). The subspecies-by-cell type interaction was not significant. If eggs were replaced, replacement was quicker in *A. m. carnica* than in *A. m. mellifera* ($P = .01$, $n = 219$) and in drone cells versus worker cells ($P = 1.80 \times 10^{-7}$, $n = 219$). In *A. m. carnica*, replacement took 36.4 ± 0.3 h for drone and 41.1 ± 2.8 h for worker cells. In *A. m. mellifera*, it took 39.9 ± 2.3 h for drone and 45.6 ± 4.2 h for worker cells (each: mean \pm SE).

Experiment 3: Replacement of Eggs at Different Time Periods after Egg Removal

Queen-laid eggs removed from drone cells 0–24 h after laying are more likely to be replaced than eggs removed 1 or 2 days later ($P = .000392$, $n = 270$; fig. 3). Forty per cent ($\pm 10\%$; mean \pm SE) of eggs removed aged 48–72 h, $53.3\% \pm 14.5\%$ removed aged 24–48 h, and $93.3\% \pm 3.3\%$ removed aged 0–24 h were replaced within 72 h. Younger eggs were also quicker replaced with $90.0\% \pm 0\%$ aged 0–24 h being replaced within 24 h versus only $36.7\% \pm 16.7\%$ aged 24–48 h and $3.3\% \pm 3.3\%$ aged 48–72 h (each: mean \pm SE). As a consequence, the increase in the proportion of eggs replaced within 72 h after egg removal shows a different pattern in young versus old eggs ($P = .000632$, $n = 270$; fig. 3).

Discussion

The results show that worker policing in the honeybee, *Apis mellifera*, has a low cost in terms of the removal of queen-laid eggs in both drone and worker cells. Experiment 1 shows that the great majority of queen-laid eggs survive: over the 2 days of the 3-day egg stage that eggs were monitored directly in their cells, survival was 90%–96% in drone and worker cells, respectively. Over the full 3 days of the egg stage survival is estimated to be 88%–94%. Experiment 2 demonstrates that most eggs that are removed are replaced and that replacement can be rapid.

The results of experiment 1 indicate that the high removal, ~50% after 24 h, of queen-laid eggs observed in previous studies of worker policing in the honeybee was due to the manipulation and relocation of eggs. This experiment involved only minimal disturbance to the eggs and therefore gives a more realistic estimate of the natural proportion of queen-laid eggs that survive. The only and inevitable disturbance to the eggs was the removal of a comb from the colony to record the presence or absence of eggs in cells. Previous research indicates, however, that any effects of our manipulation would have been negligible: queen-laid egg viability decreases only at extremely



Figure 3: Experiment 3. Cumulative replacement, by the queen laying new eggs, of eggs removed at different ages from drone cells in *Apis mellifera carnica*. Dots represent means. Vertical lines represent standard errors. Eggs removed at the age of 0–1 day were more likely to be replaced, and this happened more quickly.

low humidity (30% RH; Wegener et al. 2010). Eggs killed by suffocation with CO_2 are not more likely to be policed than untreated live eggs (Beekman and Oldroyd 2005). Eggs killed by freezing can remain for several days in combs (M. H. Kärcher, personal observation). A slight and temporary decrease in air temperature would lead only to slower egg development (Harbo and Bolten 1981).

Interestingly, a larger proportion of queen-laid eggs in drone cells were killed during the first 24-h monitoring period (eggs were aged 0–24 h at the start) compared to the second 24 h. However, this was not true for eggs in worker cells. Eggs in drone cells were also twice as likely to be removed as eggs in worker cells. One possible reason for this is provided by the theory of acceptance thresholds (Reeve 1989). Given that workers normally lay eggs in drone cells (this study; Ratnieks 1993), eggs in drone cells may be subject to a less permissive acceptance threshold leading to a greater rate of removal, in error, of queen-laid eggs in drone cells than worker cells.

Eggs removed from drone cells were replaced more quickly than eggs from worker cells. However, this probably has little effect in reducing the overall cost of policing errors (i.e., faster replacement in the type of cell with greater removal error rate) because many more workers than drones are reared. Our data are in line with the finding that the queen's tendency to replace male eggs is higher than for female eggs (Wharton et al. 2007). Eggs were

more likely to be replaced and at a quicker rate in *Apis mellifera carnica* (April and May) than in *Apis mellifera mellifera* (June and July). Future studies are necessary to determine whether this is simply due to environmental factors caused by the different study locations and conditions such as season or actual biological differences between these two subspecies. However, the overall pattern in egg replacement, as for the other experiments, was similar for both subspecies, which were both studied within their native range.

The sooner after egg laying that an egg was removed from its cell, the quicker and the more likely it was to be replaced (fig. 3). A possible reason for this is that the queen is more likely to remain or revisit this area to lay more eggs, given that honeybee queens lay eggs in large patches over several days. Given that eggs are policed between 0 and 3 days after being laid, the natural rate of egg replacement is some combination of the egg replacement rates at different egg ages until hatching, 3 days after laying. Our results show that the queen keeps relaying eggs for at least 3 days with decreasing intensity, and it is likely that the queen keeps relaying eggs for longer. In the honeybee, cells may also become vacant due to the removal of larvae of different ages or pupae (Bigio et al. 2014). For example, diploid male larvae are eaten by workers soon after hatching from the egg (Woyke 1963).

Our results from experiments 1, 2, and 3 all demonstrate that the overall cost of worker policing is very low at the colony level. Low cost is necessary to maintain a policing system. If costs in a recognition system were too high (i.e., if the policing of queen-laid eggs resulted in a high cost to the colony due to errors leading to the killing of queen-laid eggs), the best option would be an “accept all” strategy (Reeve 1989), in this case no policing of eggs. Previous studies show that only 1%–2% of worker-laid eggs are mistakenly not destroyed (Ratnieks and Visscher 1989; Ratnieks 1990b, 1993). This leads to a combined error rate of $\sim 6.2\%$ (accept 1.5% of worker-laid eggs + reject $4.1\% \times 0.9 + 9.6\% \times 0.1$ of queen-laid eggs). Applying our model, this error rate would rise to $\sim 7.9\%$ (accept 1.5% of worker-laid eggs + reject $5.7\% \times 0.9 + 12.5\% \times 0.1$ queen-laid eggs). Although not perfect, this is much better than the combined rate, 100% ($50\% + 50\%$), that arises from “guessing,” as would occur in the absence of recognition information (Ratnieks 1991).

Interestingly, Reeve’s acceptance threshold theory (model 1; Reeve 1989) is also compatible with another trend in our data: The cost of accepting a worker-laid egg (and so rearing a worker’s son related by 0.15 vs. a queen’s son related by 0.25) is greater than the cost of rejecting a queen-laid egg (the cost of killing a queen-laid egg is low, as most are rapidly replaced). As a consequence, the acceptance threshold should be set to make more errors on

queen-laid eggs (i.e., police queen-laid eggs) than worker-laid eggs (i.e., accept worker-laid eggs), as seen.

This study also provides some information on worker-laid eggs in colonies with a queen. Worker-laid eggs were infrequent but occurred, showing the need for policing. Nine of the 14 worker-laid eggs that we found in drone cells could be tracked for 1 day. Seven of them (46% weighted mean, 78% arithmetic mean) survived for 24 h, which is higher than reported by Ratnieks (1993), where only 15% (arithmetic mean) of similar-aged eggs survived for an additional 24 h. Eggs transferred with forceps have an even lower survival rate, 2% after 24 h (Ratnieks and Visscher 1989). In part, this is because these eggs were manipulated. In addition, they had not been exposed to policing before introduction into the discriminator colonies since they were taken from queenless egg-source colonies in which worker policing is switched off (Miller and Ratnieks 2001; Châline et al. 2004). The eggs we detected and monitored, however, had already been exposed to 0–24 h of worker policing in their queenright colony. The 46% of worker-laid eggs that survived from the second to third comb inspections is not significantly different from the survival of 0–24-h-old queen-laid eggs to their next inspection. This indicates that once a worker-laid egg has survived for a certain period of time, 0–24 h, it is more likely to survive the next 24 h. Possibly, some worker-laid eggs are harder to recognize, and these are the ones that are not policed, comparable with the situation in anarchistic colonies (Oldroyd and Ratnieks 2000). Our data also indicate that low removal rates for worker-laid eggs may also occur in colonies that are not overtly anarchistic. Approximately 12.7 times as many worker-laid eggs per drone cell were found in the *A. m. mellifera* than in the *A. m. carnica* study. Further investigation would be needed to determine whether this represents a consistent difference among subspecies.

Previous studies of worker policing in the honeybee have focused mainly on the benefits to the policing workers in the killing of worker-laid eggs. This benefit is great as workers are more related to their mother queen’s sons (0.25) than to sister workers’ sons (0.15, assuming an effective paternity of 10). By showing that one major potential cost of policing, the killing in error of queen-laid eggs, is low, this study indicates that worker policing in the honeybee is favored due to both high benefit and low cost. This conclusion is probably a broad one and is in agreement with data from vespine wasps. In three wasp species, video analyses have revealed that most unmanipulated queen-laid eggs were not policed during the first day after laying: 96% survival for *Dolichovespula sylvestris* (Wenseleers et al. 2005b), 98% for *Vespula rufa* (Wenseleers et al. 2005a), and 92% for *Dolichovespula norwegica* (Bonckaert et al. 2011). In three other vespine species, queen-

laid eggs were experimentally transferred and had a high removal rate: *Vespula vulgaris* (Foster and Ratnieks 2001a), *Vespa crabro* (Foster et al. 2002), and *Vespula germanica* (Bonckaert et al. 2008). This study means that the honeybee is now the only species in which we know the survival of both manipulated and unmanipulated queen-laid eggs, in which a model was added to estimate real egg survival of unmanipulated queen-laid eggs, and in which we have good knowledge of both the costs and benefits of worker policing.

Acknowledgments

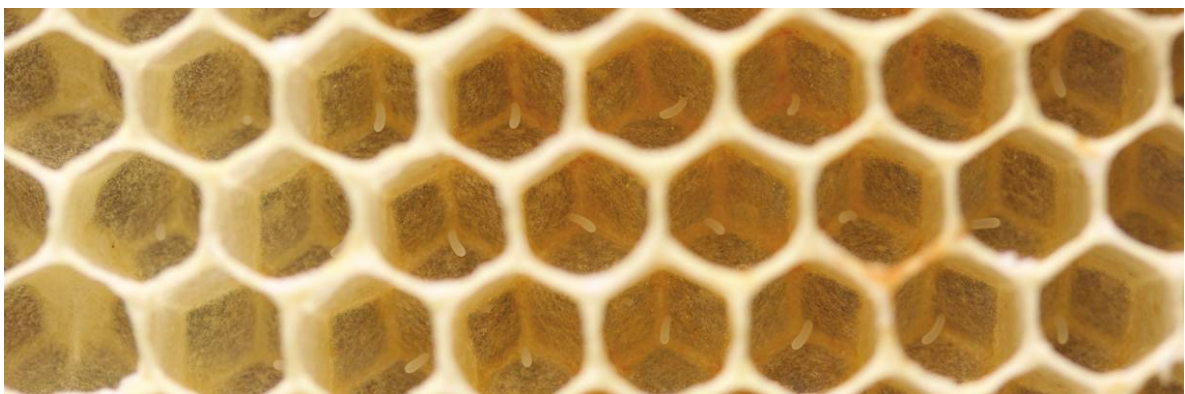
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Eggs recently laid by the queen in cells of a naturally built drone comb. Photo credit: M. H. Kärcher.